

IMMUNOHISTOLOGICAL LOCALIZATION OF SEROTONIN IN THE CNS AND FEEDING SYSTEM OF THE STABLE FLY *Stomoxys calcitrans* L. (DIPTERA: MUSCIDAE)

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Serotonin, or 5-hydroxytryptamine (5-HT), plays critical roles as a neurotransmitter and neuromodulator that control or modulate many behaviors in insects, such as feeding. Neurons immunoreactive (IR) to 5-HT were detected in the central nervous system (CNS) of the larval and adult stages of the stable fly, Stomoxys calcitrans, using an immunohistological technique. The location and pattern of the 5-HT IR neurons are described and compared for these two different developmental stages. Anatomical features of the fly feeding system were analyzed in third instar larvae and adult flies using a combination of histological and immunohistological techniques. In third instar larvae, the cibarial dilator muscles were observed within the cibarial pump skeleton and innervated by 5-HT IR neurons in nerves arising from the brain. There were four

Grant sponsor: USDA, ARS, Postdoctoral Research Associate Program Award.

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ARCHIVES OF INSECT BIOCHEMISTRY AND PHYSIOLOGY, Vol. 77, No. 4, 199–219 (2011)

Published online in Wiley Online Library (wileyonlinelibrary.com).

© 2011 Wiley Periodicals, Inc. DOI: 10.1002/arch.20434

pairs of nerves arising from the frontal surface of the larval brain that innervate the cibarial pump muscles, pharynx, and muscles controlling the mouth hooks. A strong serotonergic innervation of the anterior stomatogastric system was observed, which suggests 5-HT may play a role in the coordination of different phases of food ingestion by larvae. Similarly, many 5-HT IR neurons were found in both the brain and the thoracico-abdominal ganglia in the adult, some of which innervate the cibarial pump dilator muscles and the stomatogastric muscles. This is the first report describing neuromuscular structures of the stable fly feeding system. The results reported here suggest 5-HT may play a critical role in feeding behaviors of stable fly larvae and adults. © 2011 Wiley Periodicals, Inc.

Keywords: biting fly; feeding system; cibarial pump; 5-hydroxytryptamine; neuroanatomy

INTRODUCTION

The stable fly, *Stomoxys calcitrans* (L.) (Diptera: Muscidae), is one of the most significant blood-feeding flies affecting animals, including cattle, horses, and dogs, as well as humans. Larvae and adults of the stable fly differ in their feeding habits. Larvae feed on a solid diet made of cattle manure, hay, and other waste materials on the ground, whereas the adults are obligate blood feeders of animals and humans. Feeding behaviors and the physiological and molecular mechanisms that control feeding have been investigated in several invertebrate models, including the pond snail, *Lymnaea stagnalis* (Rose and Benjamin, 1981; Staras et al., 1998; Straub et al., 2002), and the sea slug, *Aplysia californica* (Rosen et al., 2000; Evans et al., 2005; Wentzell et al., 2009). Feeding musculature, neuronal circuits involved in the control of feeding, and the pharmacological modulation of feeding behaviors have also been investigated in detail in a few insect species, including locusts (Rast and Bräunig, 1997, 2001; Zilberstein and Ayali, 2002; Ayali, 2004), the adult tobacco hornworm (Miles and Booker, 1998; Ayali, 2004), the adult blowfly (Gelperin, 1967, 1972; Fredman and Steinhardt, 1973; Starre and Ruigrok, 1980; Mier et al., 1985; Bowdan and Dethier, 1986), and blowfly larvae (Schoofs and Spieß, 2007; Schoofs et al., 2009). Feeding by insect larvae with a head capsule and mouthparts involves rhythmic movements of the mandibles, pharynx, and esophagus (Miles and Booker, 1994, 1998), whereas feeding is achieved through rhythmic protraction and retraction of the cephalo-pharyngeal skeleton (CPS) and associated shoveling movements of the mouth hooks in calyptrate dipteran larvae without head capsule (Roberts, 1971; Schoofs et al., 2009). Blowfly larvae ingest food by contraction of the cibarial dilator muscles (CDM) that are located inside the CPS (Schoofs and Spieß, 2007; Schoofs et al., 2009). Neither the musculature of the stable fly feeding system nor neuronal control of the cibarial pump has been previously studied in the stable fly. Understanding the mechanisms of feeding in both larval and adult stages of this biting fly species would enable the discovery of new molecular targets for the development of novel fly control technologies.

Biogenic amines, including serotonin (5-hydroxytryptamine or 5-HT), are important neurotransmitters and neuromodulators regulating many essential physiological processes (Nässel, 1988). 5-HT immunoreactive (IR) neurons have been located

in the central nervous system (CNS) and peripheral nervous system of many insect species, including the horse fly (*Tabanus nigrovittatus*) (Haselton et al., 2006), the dragonfly (*Epiptera* spp. and *Pachydiplax longipennis*) (Longley and Longley, 1986), the yellow fever mosquito (*Aedes aegypti*) and the African malaria mosquito (*Anopheles gambiae*) (Moffett and Moffet, 2005; Siju et al., 2008). 5-HT affects feeding preference, meal size, and feeding-related activities in the black blow fly, mosquitoes, the locust, the flesh fly, and the *Rhodnius* bug (Long and Murdock, 1983; Novak and Rowley, 1994; Novak et al., 1995; Dacks et al., 2003; Molaei and Lange, 2003; Orchard, 2006). 5-HT was shown to play a critical role in regulating the feeding activity of aphids and whiteflies (Flückiger et al., 1992; Harrewijn and Kayser, 1997). Pymetrozine is a novel insecticide that kills whiteflies and aphids by interfering with the serotonergic system involved in their feeding behavior (Harrewijn and Kayser, 1997; Kaufmann et al., 2004).

Here, we describe the localization of 5-HT immunoreactivity in the CNS and systems related to feeding processes in the stable fly. We also describe the muscular organization, and innervations of serotonergic neurons of the cibarial pump in the larval and adult stages of the stable fly.

MATERIALS AND METHODS

Flies

Stable flies were obtained from a laboratory colonized stable fly strain maintained at the USDA, ARS, Knippling-Bushland US Livestock Insects Research Laboratory in Kerrville, TX. The fly colony was maintained at $27.8 \pm 1^\circ\text{C}$, $60 \pm 1\%$ RH, and a photoperiod of 12:12 L:D. Larvae were reared in a Purina Fly Larvae Media (PharmaServ, Framingham, MA) covered with peanut hull pellets (from local livestock feed mills), whereas adult male and female flies were held in a solid bottom screen cage, and fed daily with citrated bovine blood supplied in saturated cotton pads. The citrated bovine blood contained 6.6 g sodium citrate, 264,000 units nystatin, and 264 mg kanamycin sulfate per liter.

Tissue Preparations

To characterize the fine structure of the stable fly feeding systems and visualize 5-HT IR neurons in the CNS and the innervation of cibarial pump muscles by serotonergic neurons, third instar larvae and adult flies (2–8 days after eclosion) were chilled at 4°C for 30 min in a refrigerator before dissection. Thereafter, the insects were pinned, without damaging the related structures and tissues, in a Petri dish containing black Silgard[®] silicone. Larvae were placed dorsal side up and opened along the lateral midline with a pair of fine scissors. Adults were pinned ventral side up in the Petri dish and cut open along the ventral midline. All dissections were made in cold stable fly physiological saline under a Wild Heerbrugg dissection microscope (Gais, Switzerland). Stable fly saline contained 105 mM NaCl, 5 mM KNO_3 , 5 mM CaCl_2 , 3 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mM L-histidine, and 28 mM glucose (pH 6.8) (Cook, 1992).

Five to 10 specimens, including one control, for each tissue type, such as the brain and cibarial pump, were processed for general histology and immunohistology analyses. Rabbit anti-5HT (Immunostar, Inc., Hudson, WI) was used as the primary antibody for immunohistochemistry and was detected using Alexa Fluor 488-conjugated donkey

anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA). In control preparations, the primary antibody was omitted and only the secondary antibody was included to verify the specificity of labeling as well as to assess background noise. Control preparations did not show any specific labeling in the target tissues. In order to better distinguish neuronal labeling from auto-fluorescence emitted by fly musculature, double labeling with Fluor 546 Phalloidin (Invitrogen) for F-actin staining was also included in the 5-HT immunohistochemistry protocol.

Fine Structure Characterization

Following dissection, insect preparations with the CNS and feeding-related systems or tissues exposed were examined under a Meiji Techno EMZ 13TR microscope (Meiji Techno America, Santa Clara, CA). In order to better visualize the fine structures, a drop of 1% methylene blue chloride or neutral red (Scholar Chemistry, Avon, NY) was applied onto dissected tissues to help distinguish nerves and muscles. After 30 sec, the tissues were rinsed with saline solution and then photographed using a Pixelink FireWire digital camera mounted on the microscope (Aegis Electronic Group, Inc., Gilbert, AZ). Digital images were captured using Pixelink software. Schematic diagrams were prepared using CorelDraw software (Corel Corporation, Ottawa, Ont., Canada).

Serotonin (5-HT) Immunohistochemistry

The immunohistochemistry protocol used to detect 5-HT immunoreactivity in stable fly tissues was modified from Davis et al. (1996) and Siju et al. (2008). Fly tissues were fixed at 4°C overnight with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Subsequently, fly tissues were washed 6 times at room temperature (RT) on a rotator for 1 h each in 0.1 M phosphate buffered saline containing triton X-100 (PBST: 136.9 mM NaCl, 2.7 mM KCl, 4.5 mM Na₂HPO₄*7H₂O, and 1.5 mM KH₂PO₄ in distilled H₂O with 0.5% Triton X-100, pH 7.2–7.4). Fly tissues were blocked at RT for 1 h in 5% normal donkey serum (NDS) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBST with 0.05% sodium azide (PBSAT), and were incubated at RT overnight in a 1:1,500 dilution of rabbit anti-5-HT (Immunostar) in blocking solution. Thereafter, the tissues were washed 5 times at RT on a rotator for 1 h each in PBST. They were blocked again in 5% NDS in PBSAT at RT for 1 h, and then incubated at RT in the dark overnight in a 1:400 dilution of donkey anti-rabbit AlexaFluor 488 (Invitrogen) in the blocking solution. This was followed by washing the tissues at RT under darkness on a rotator for 1 h in PBST, and 30 min in 0.1 M PBS. Finally, tissue preparations were cleared for 15 min each in 40, 60, and 80% glycerol, mounted in 80% glycerol, and stored at 4°C before microscope examination and imaging.

Plastic Embedding

Tissues used for cibarial pump immunohistochemistry studies were dissected in stable fly saline and embedded in Spurr's plastic resin (Electron Microscopy Sciences, Hatfield, PA). Spurr's embedding medium was prepared by mixing the following ingredients for 10 min in a plastic cup under a fume hood: 26.0 g nonenyl succinic anhydride, 10.0 g vinyl cyclohexene dioxide, 6.8 g diglycidyl ether, and 0.4 g dimethylaminoethanol. The 5-HT immuno-histochemistry procedure, including incubations in the first and secondary antibodies, was the same as described above. After washing at RT in darkness on a rotator for 1 h in PBST and 30 min. in 0.1 M PBS,

and the specimens were then sequentially dehydrated for 10 min in each of the following: 30, 50, 70, 90, 96, 100% ethanol (twice), and distilled acetone (twice). Tissue specimens were then incubated in 1:1 Spurr's resin:acetone and 100% Spurr's for 2 and 4 h, respectively, on a rotator under darkness. Thereafter, the specimens were placed in a Beem[®] capsule with fresh Spurr's resin and polymerized for 12 h at 60°C in an incubator. Specimens embedded in Spurr's were sectioned at a thickness of 14 µm using a microtome (Erma Inc., Tokyo, Japan), mounted in a fluorescent mounting medium (Fluoromount, Serva, Germany) on Platinum Line microscope slides (Knittel Glaeser, Braunschweig, Germany), and stored at 4°C before imaging.

Double Labeling

For the 5-HT/F-actin double labeling, the procedure was the same as above except for fixation and incubation of the secondary antibody. Tissues were exposed to cold HEPES-buffered saline (HBS; 150 mM NaCl, 5 mM KCl, 5 mM CaCl₂, 25 mM sucrose, 10 mM HEPES (*N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulphonic acid])) (Heidel and Pflüger, 2006; Ott, 2008)) and fixed in zinc-formaldehyde (4%) overnight at 4°C. The zinc-formaldehyde fixative contained 18.4 mM ZnCl₂, 135 mM NaCl, 35 mM sucrose, and 4% formaldehyde in distilled water (pH 6.35) (Ott, 2008). Tissues were washed for 1 h in HBS before being washed twice for 1 h each in 0.1 M PBST on a rotator at RT. After the tissues were incubated with the primary antibody (rabbit anti-5-HT) and blocked in 5% NDS in PBSAT (200 µl) for 1 h, a volume of 2.5 µl donkey anti-rabbit Alexa Fluor 488 (Invitrogen) and 5 µl Alexa Fluor 546 Phalloidin (Invitrogen) were added to the blocking solution (200 µl). Tissue blocks were incubated overnight on a rotator under darkness. Tissues were dehydrated and rehydrated in graded ethanol for 10 min in each of the following: 50, 70, 90, 100, 100, 90, 70, and 50%.

For the 5-HT/neurobiotin double labeling, test flies were fed with 0.25% neurobiotin (Vector Laboratories, Inc., Burlingame, CA) in citrated bovine blood (50 µl 1% neurobiotin + 200 µl bovine blood with sodium citrate) for 4 h for mouthpart studies. The flies were then dissected and tissues were fixed and washed as described for 5-HT protocol. After the tissues were incubated with the primary antibody (rabbit anti-5-HT) and blocked in 5% NDS in PBSAT (200 µl) for 1 h, a volume of 1 µl Cy3 conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc.) and 5 µl Avidin Alexa Fluor 488 conjugate (Molecular Probes, Inc., Eugene, OR) were added to the blocking solution (200 µl). Tissue blocks were incubated overnight on a rotator under darkness. Tissues were then washed in PBST and PBS, and cleared in glycerol and mounted on slides as described for 5-HT procedure.

Imaging

Tissue slides were examined under an Olympus BX60 fluorescence microscope (Olympus America Inc., Center Valley, PA), and the digital color images of tissues were documented using an Olympus DP70 digital camera and associated software attached to the fluorescence microscope. Digital images were processed for contrast and brightness using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA).

Some tissue slides were further imaged using a Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY). Alexa Fluor 488 (for 5-HT) and Alexa Fluor 546 Phalloidin (for F-actin)-labeled tissues were excited using a 488 nm Argon/2 laser and a 543 nm HeNe (Helium–Neon) laser, respectively. The z-stack interval was 1 µm. Images of emitted fluorescence from 5-HT

and F-actin immunoreactivity were collected and analyzed using Axiovision (Carl Zeiss Microimaging, Jena, Germany) and Image J (NIH, Bethesda, MD) software.

RESULTS

Structure and Muscular Organization of the Larval Feeding System, CNS and Frontal Portion of Digestive System

A schematic drawing of structures related to the feeding, CNS and digestive systems of third instar larvae of the stable fly is presented in Figure 1. The drawing is based on histological analysis of the feeding-related systems using general nerve/muscle staining and light microscopy techniques. Stable fly larvae are acephalic (without head capsule). As such, the larval feeding system comprises the mouthparts with a mouth hook at the front of the larva and the CPS (Figs. 1 and 2A), which is an elongated brownish

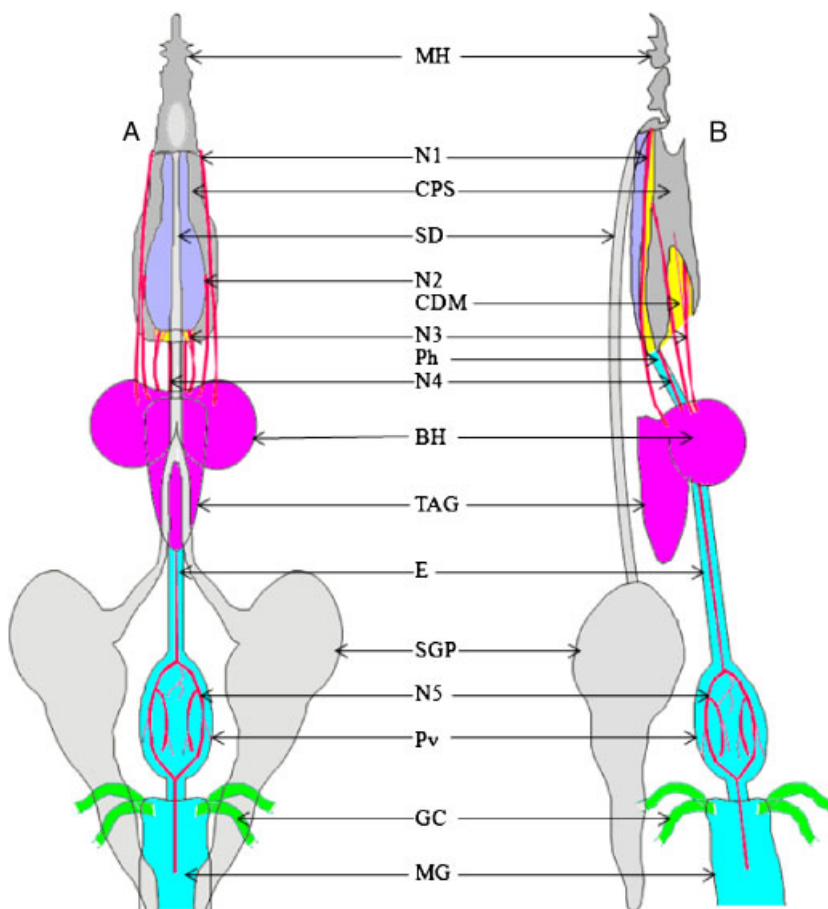


Figure 1. Schematic drawing of fine structures and nerves related to the feeding system of third instar stable fly larvae. (A) A ventral view. (B) A lateral view. BH, brain hemispheres. CDM, cibarial dilator muscles. CPS, cephalo-pharyngeal skeleton. E, esophagus. GC, gastric caeca. MG, midgut. MH, mouth hook. N1, N2, N3, N4, and N5, Nerves. Ph, pharynx. Pv, proventriculus. SD, salivary duct. SGP, salivary gland pouch. TAG, thoracico-abdominal ganglion.

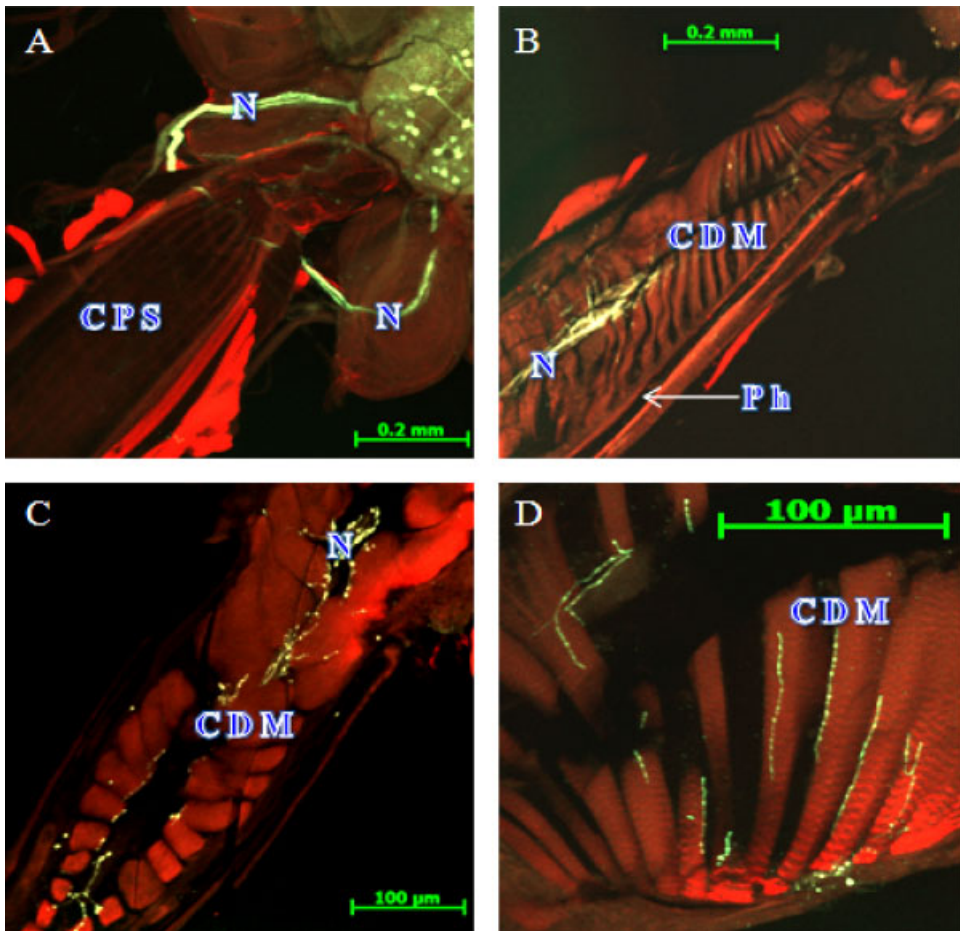


Figure 2. Confocal images of 5-HT/F-actin double labeling in the cibarial pump of stable fly third instar larvae. (A) Whole mount confocal micrograph of 5-HT IR nerves (N) extending from the CNS to cibarial pump. (B) Confocal image of cibarial dilator muscles with 5-HT IR nerves (N) above the pharynx. (C) Confocal image of 5-HT IR nerves (N) extending to each cibarial dilator muscle inside the cibarial pump. (D) Confocal image of 5-HT IR innervations in cibarial dilator muscle fibers. CDM, cibarial dilator muscles. CPS, cephalo-pharyngeal skeleton. N, nerves. Ph, pharynx.

structure in front of the brain. The larval CPS moves back and forth repetitively during feeding. Inside the CPS, there is a parallel array of approximately 20–30 pairs of CDM (Fig. 2B and C), which are attached to the CPS walls. As is typically observed with insect muscles, the CDMs are striated (Fig. 2D). The CDMs are innervated by neurons from a pair of nerves arising from the posterior end of the larval brain (Figs. 1 and 2B, C, D). The larval CNS is composed of two brain hemispheres and a thoraco-abdominal ganglion (TAG). Parts of the anterior digestive system include the pharynx, esophagus, salivary gland, and proventriculus (Fig. 1). Four pairs of fine nerves arise from the brain hemispheres: one pair innervates the CDM (N3), another pair travels forward along the pharynx (N4), and two pairs extend to muscles outside the CPS that may control the mouth hook (N1, N2). Each nerve has delicate branches and their endings are difficult to track. The stomatogastric nerves (N5) also arise from the larval brain hemispheres, and innervate the proventriculus (Figs. 1, 3B and 4A).

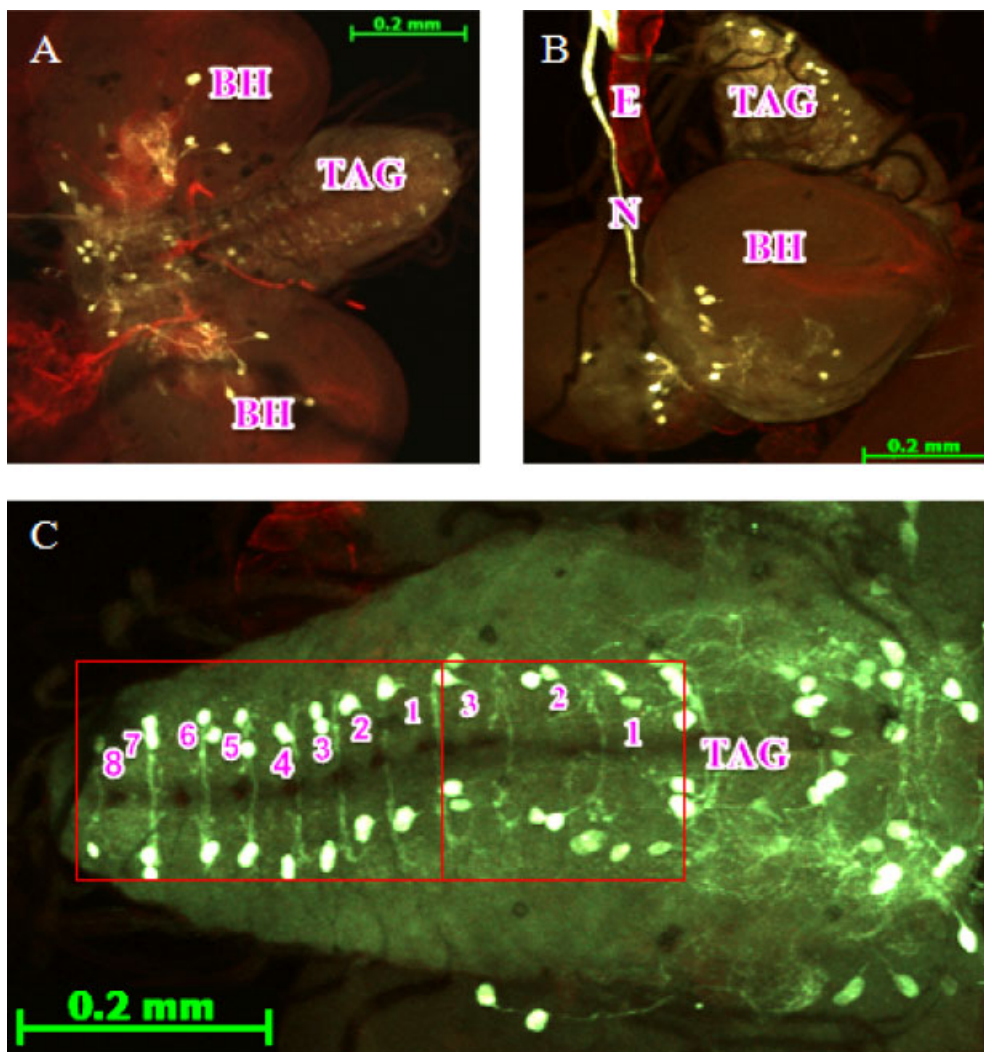


Figure 3. Confocal Z-projections showing 5-HT/F-actin double labeling in the CNS of stable fly third instar larvae. (A) A dorsal view displaying 5-HT IR neuronal processes. (B) A lateral view showing three-dimensional distributions of 5-HT IR neuronal cells. (C) A ventral view showing 5-HT IR neuronal cells and processes in the abdominal neuromere (rectangle) and in the thoracic neuromere (square). BH, brain hemispheres. E, esophagus. N, nerves, connecting to the stomatogastric nervous system. TAG, thoracic-abdominal ganglion.

Distributions of 5-HT IR Neurons in the Larval CNS

A notable feature of the larval CNS of the stable fly is the wide distribution of 5-HT IR neurons and neuronal processes. Between 80 and 90 bilaterally symmetrical 5-HT IR neurons were found in the brain hemispheres and the TAG of third instar stable fly larvae (Fig. 3). In the brain hemispheres some neuronal processes are clearly seen near the brain surface, whereas others are found deep inside the brain (Fig. 3A and B). There are 20–25 5-HT IR neurons in each of the brain hemispheres. The TAG contains 40–50 5-HT IR neurons. A total of eight bilaterally symmetrical clusters of

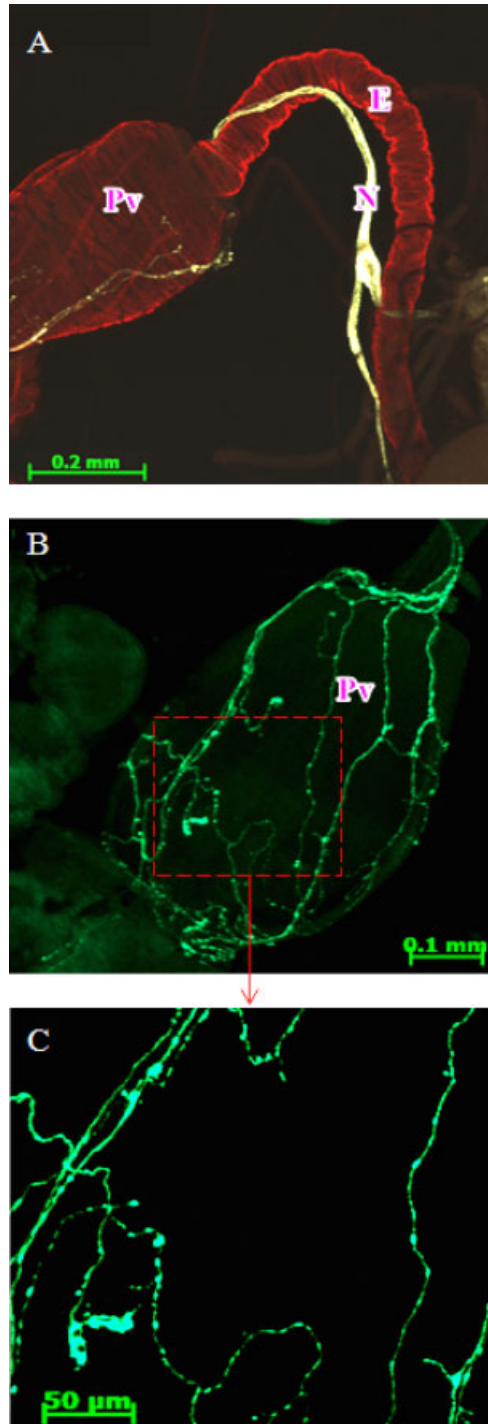


Figure 4. Confocal images showing 5-HT or 5-HT/F-actin double labeling in the foregut of third instar larvae whole mount. (A) 5-HT/F-actin double labeling immunoreactive nerves from the CNS backward to the proventriculus along with the esophagus. (B) 5-HT IR nerves around the proventriculus. (C) Enlarged image from the boxed area in B. E, esophagus. N, nerves. Pv, proventriculus.

5-HT IR neurons were present in the anterior-lateral margin of the ganglion (Fig. 3C rectangle). Each of these eight neuron clusters occupies one segment of the TAG. In the mid-posterior area of the larval TAG, there are three groups of 5-HT IR neurons that occur bilaterally symmetric and send their processes into the neuropile (Fig. 3C square).

5-HT IR Neuronal Innervations of Larval CDMs and Anterior Digestive System

Serotonergic innervations of the CDM were observed in third instar stable fly larvae. A pair of 5-HT IR nerves arise from the CNS sending several main branches into the CPS (Fig. 2A). These main nerve branches divide further into fine processes that innervate each of the 20–30 CDMs (Fig. 2B and C). The terminal-like varicosities of the 5-HT IR neuronal processes along the muscle fibers indicate sites of neuromuscular junctions (Fig. 2D).

Serotonin (5-HT) IR nerves arising from the larval brain project posteriorly along with the esophagus and foregut to the proventriculus (Fig. 4A and B). The proventriculus, localized between the esophagus and midgut, was innervated by fine processes of the 5-HT IR neurons that had many varicosities, suggesting these are sites of serotonin release at neuromuscular junctions (Fig. 4C). Extensions of the 5-HT IR neuronal processes from the proventriculus to the anterior portion of midgut were also noted (Fig. 4B).

Structure and Muscular Organization of the Adult CNS, Feeding System, and Frontal Portion of the Digestive System

Schematics of the fine structures associated with the CNS and feeding-related systems of the adult fly from the ventral and lateral views are presented in Figure 5. The drawing is again based on histological analysis of the feeding-related systems using general nerve/muscle staining and light microscopy techniques. The confocal images (Figs. 6–9) show the CNS, mouthparts, gut, and nerve distributions. The larval salivary glands were observed to be pouch-like in appearance, whereas the adult salivary glands were tube shaped (Figs. 1 and 5).

The Adult CNS and Distribution of the Distribution of 5-HT IR Neurons

In the adult stable fly, the CNS is composed of the brain and a thoracic-abdominal ganglion (TAG), and the connectives connecting the brain and the TAG (Figs. 5, 6 and 7). Brain structures include two optic lobes (OpL), one pars intercerebralis (PI), one central complex (CeC), two olfactory lobes (OIL), and the subesophageal ganglion (SG) (Fig. 6). The TAG is fused structure that is composed of all thoracic and abdominal neuromeres (Fig. 7). This is consistent with findings in other higher Diptera (Chapman, 1998).

Serotonin (5-HT) IR neurons were observed in bilateral pairs throughout the brain and neuronal processes projected to neuropils in different brain areas. The total number of 5-HT IR neurons in the adult brain was estimated at between 86 and 132. However, variation in the total number of 5-HT IR neurons existed among individual flies. Serotonin IR neurons in the brain of the adult stable fly were found in the lateral and median regions of the superior PI and CeC with neuronal processes extending into the central area when viewed from the posterior end of the brain (Fig. 6A). There are 50–90 5-HT IR neurons in the PI and CeC areas. Serotonin IR neurons were found in the middle and lower proximal regions at the base of the OpL and in the SG.

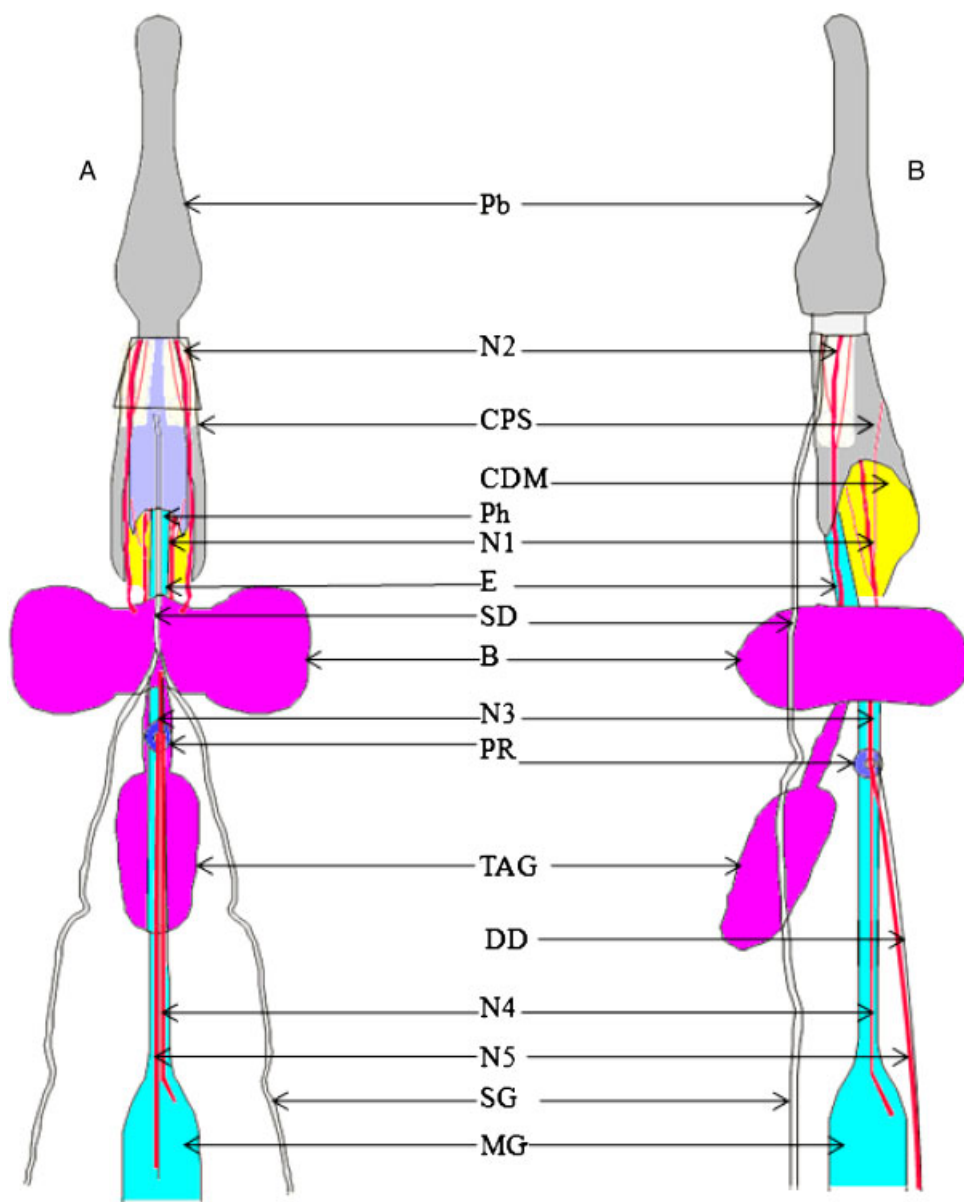


Figure 5. Schematic drawing of fine structures and nerves related to the feeding system of adult stable flies. (A) A ventral view. (B) A lateral view. B, brain. CDM, cibarial dilator muscle. CPS, cephalo-pharyngeal skeleton. DD, divericulum duct. E, esophagus. MG, midgut. N1, N2, N3, N4 N5, Nerves. Pb, proboscis. Ph, pharynx. PR, proventriculus ring. SD, salivary duct. SG, salivary gland. TAG, thoracic-abdominal ganglion.

Processes of 5-HT IR neurons travel along the cervical connectives to reach the TAG (Figs. 6B and 7A). A group of 16–22 5-HT IR neurons was observed in the SG region. Each OpL contained up to 20 5-HT IR neurons. 5-HT IR neuronal connections were observed between CeC and OpLs (Fig. 6A and B).

Serotonin (5-HT) IR neuronal processes arising from the cervical connectives reached the TAG to form a network that covered the dorsal surface of the TAG.

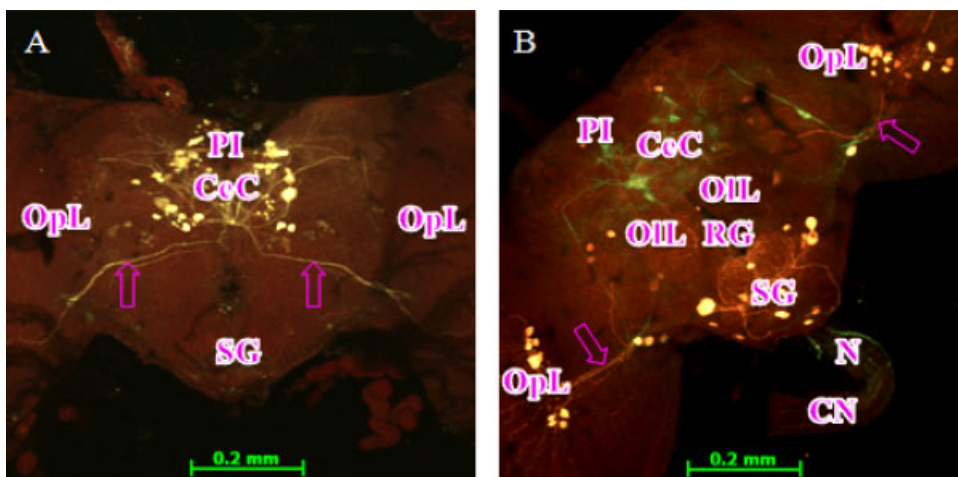


Figure 6. Confocal Z-projection of 5-HT/F-actin double labeling in whole mounts of the adult stable fly brain. (A) A posterior view of the brain depicting the distribution of 5-HT IR neurons. (B) An anterior view of the brain depicting the distribution of 5-HT IR neurons. Arrow, 5-HT IR neuronal connections. CeC, central complex. CN, cervical connective. N, nerve. OIL, olfactory lobe. OpL, optic lobe. PI, pars intercerebralis. RG, ring gland. SG, subesophageal ganglion.

(Fig. 7A) and projected to the abdominal neuromeres. There were 12 individual 5-HT IR neurons along the central midline region of the three fused thoracic neuromeres (Fig. 7B). Clusters of 5-HT IR neuronal branches were noted in the front of the ganglion in the abdominal neuromeres (Fig. 7C and D), and their fine processes penetrated deep into the ganglion neuropils (not shown). Each neuromere contained inputs from a group of 5-HT IR neurons.

The Adult Feeding System and Innervations of the Cibarial Pump Muscles by 5-HT IR Neurons

The cibarial pump, a key part of the fly feeding system, has 70–100 muscles on each side of the cibarial pump which is located above the pharynx (Fig. 8A). From the brain, there are two pairs of nerves that branch forward to inside (Fig. 8A N1) and outside (Fig. 8A N2) of the cephalo-pharyngeal skeleton. Paired bundles of CDM from two groups are attached to the anterior surface of the pharynx (Fig. 8B and D). Serotonin (5-HT) IR nerves arising from the brain projected anteriorly to inside the CPS, and fine 5-HT IR neuronal processes innervated the cibarial muscles (Fig. 8C and D). In contrast to what was observed for the neuronal processes that innervated the cibarial muscles in the immature stage, no varicosity-like structure was observed in the neuronal processes that innervated the cibarial muscles in adult flies. This may suggest the serotonergic neuronal processes in adult flies may be dendrites and may serve as part of the feedback component in the feeding-related neuronal circuits in the brain.

5-HT IR Neuronal Innervation of the Anterior Digestive System

The foregut components of adult stable flies include a diverticulum duct (DD) and a proventriculus ring (PR), which is located between the pharynx and esophagus of the midgut (Fig. 9). There are two 5-HT IR nerve bundles arising from the PR (Fig. 9). One ran down the diverticulum duct and the other, consisting of stomatogastric nerve

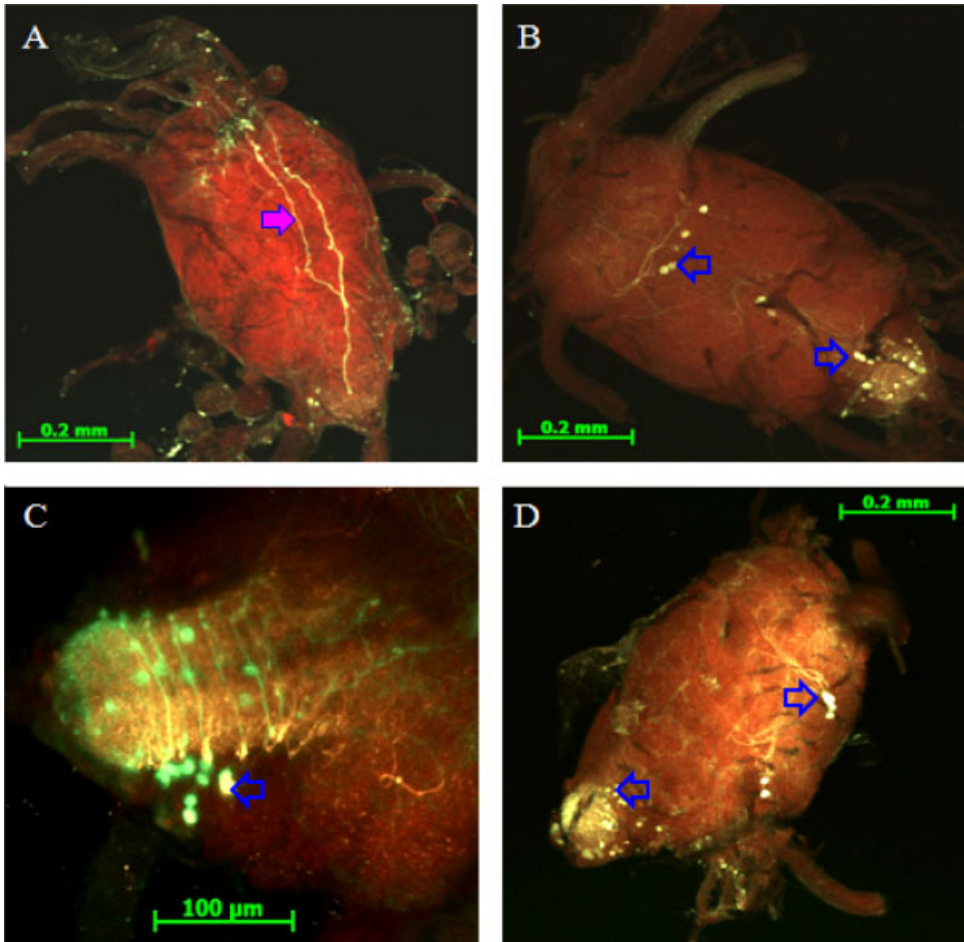


Figure 7. Confocal Z-projection of 5-HT/F-actin double labeling in adult stable fly TAG whole mounts. (A) A dorsal view of the TAG. (B) A ventral view of the TAG. (C) Enlarged abdominal neuromeres in the TAG. (D) A lateral view of the TAG. Empty arrows, 5-HT IR cell bodies of neuronal processes. Filled arrow, 5-HT IR nerves.

processes, projected posteriorly along the surface of the anterior midgut (not shown). Further, the DD, which arises from the PR, ended with a bi-lobed sac that makes contact with the midgut (not shown).

DISCUSSION

The results presented here constitute the first detailed report of 5-HT immunoreactivity in the CNS and feeding-related systems of the stable fly. This study provides a detailed report of the neuroanatomy of the cibarial pump, which is localized within the head of stable flies and plays a crucial role in the mechanics of food ingestion. Our descriptions extend to the 5-HT neuronal processes in fine structures of the CNS and feeding-related systems of the stable fly larval and adult life stages. Divergent feeding strategies evolved between the larval and adult stages of the stable fly to exploit

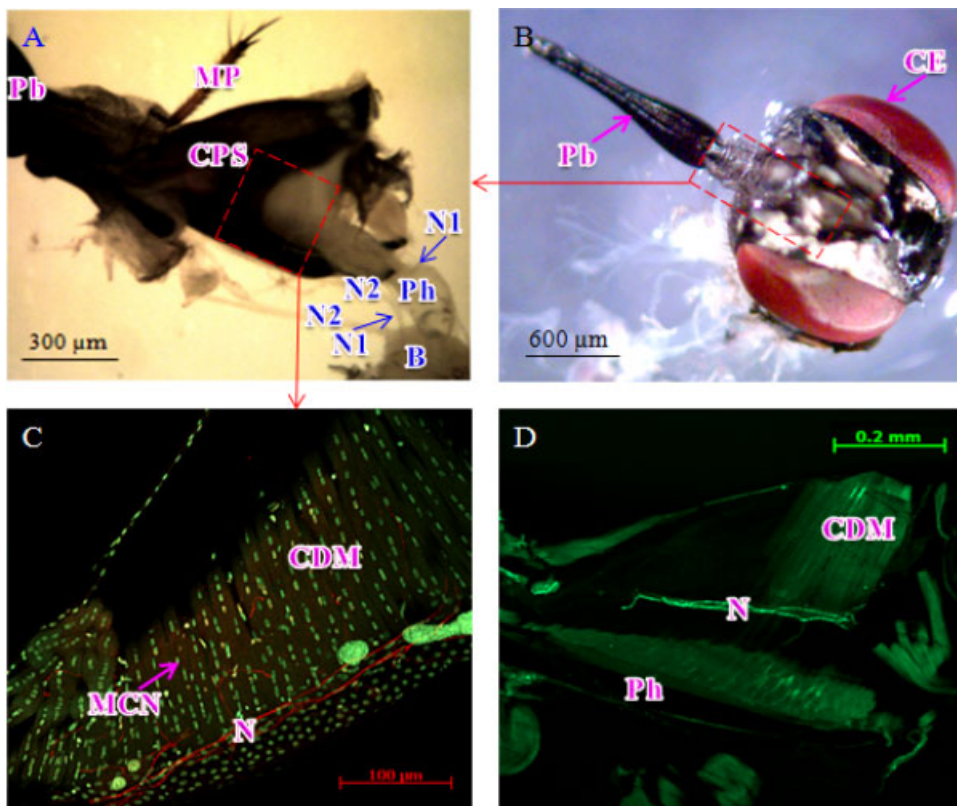


Figure 8. Cephalo-pharyngeal skeleton of the stable fly adult with the cibarial dilator muscle fibers and 5-HT IR innervations. (A) Two pairs of nerves from the brain forward to cibarial dilator muscles (N1) and outside cephalo-pharyngeal skeleton and proboscis (N2). (B) External cephalo-pharyngeal skeleton of the stable fly. (C) Confocal image of 5-HT IR neuronal processes (red, Cy-3 secondary antibody) innervating the cibarial dilator muscle fibers (green, Alexa Fluor 488). (D) the cibarial dilator muscles with 5-HT IR innervations. B, brain. CDM, cibarial dilator muscles. CE, compound eye. CPS, cephalo-pharyngeal skeleton. MCN, muscle cell nucleus. MP, maxillary palp. N1 and N2, nerves. Pb, proboscis. Ph, pharynx.

different ecological niches. Although larvae rely on nutrient sources on the ground (Foil and Hogsette, 1994), blood-feeding is essential for adult stable fly viability and reproduction (Venkatesh and Morrison, 1980; Sutcliffe et al., 1993). This work represents the first description of feeding-related structures from the stable fly.

Comparative Feeding Neuroanatomy and Physiology

Insect feeding behaviors are complex (Simpson et al., 1989). We used the term “feeding-related systems” to denote anatomical features like mouthparts, nerves, and digestive organs working in unison to acquire and coordinate the processing of food, whereas feeding describes the act of ingesting and moving nutrients through the mouthparts and on to the crop or gut. Significant structural changes were observed in the feeding-related systems between immature and adult stable flies. It is hypothesized that these structural variations reflect differences in adaptations to access food sources in disparate ecological niches, and physiological requirements to digest uncommon foods. The consideration of structure–function relationships is important in this

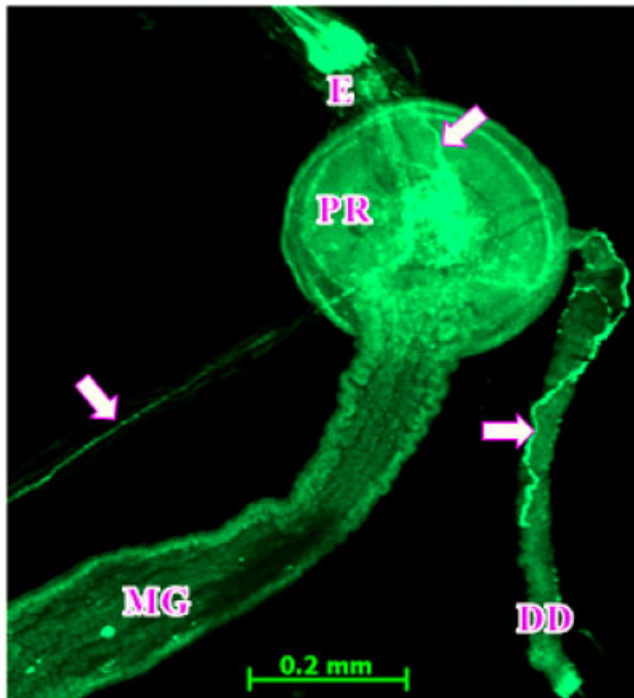


Figure 9. Confocal image of the proventriculus ring and diverticulum duct with 5-HT IR neuronal processes in the adult stable fly. Arrow, pointing to nerves, which originate in the brain and extend through the ring to the midgut and diverticulum. DD, diverticulum duct. E, esophagus. MG, midgut. PR, proventriculus ring.

regard. In stable fly larvae the proventriculus is a bulb-like structure between the foregut and the midgut, whereas in adults it is ring-like, hence our use of the term proventriculus in stable fly larvae and PR in stable fly adults. Equivalent structures were called cardia in blow fly larvae (Boonsriwong et al., 2007), and proventriculus in blow fly adults (Haselton et al., 2008).

5-HT IR Patterns in the CNS

The specificity of the primary antibody rabbit anti-5-HT (Immunostar) we used in this study has been validated in previous studies (Sykes and Condron, 2005; Chen and Condron, 2008). Results from a current study on octopamine immunoreactivity using an anti-octopamine primary antibody revealed a very different neuronal labeling pattern in the CNS of the stable fly, suggesting that the primary anti 5-HT antibody was specific to serotonin but not to octopamine or other biogenic amines (Li, unpublished).

In comparison with previous studies on other fly species (Valles and White, 1987; Haselton et al., 2006), a common feature found in the stable fly CNS was the presence of approximately 86–132 5-HT IR neurons positioned bilaterally in a symmetrical fashion. The exact number of 5-HT IR neurons may differ between flies. There were 21–25 5-HT IR neurons with their branching arborizations located in each larval brain hemisphere (Fig. 3A and 10A). However, in the adult brain the 5-HT IR neurons were separated into different functional clusters (Fig. 6 and 10B). Areas of the PI and the

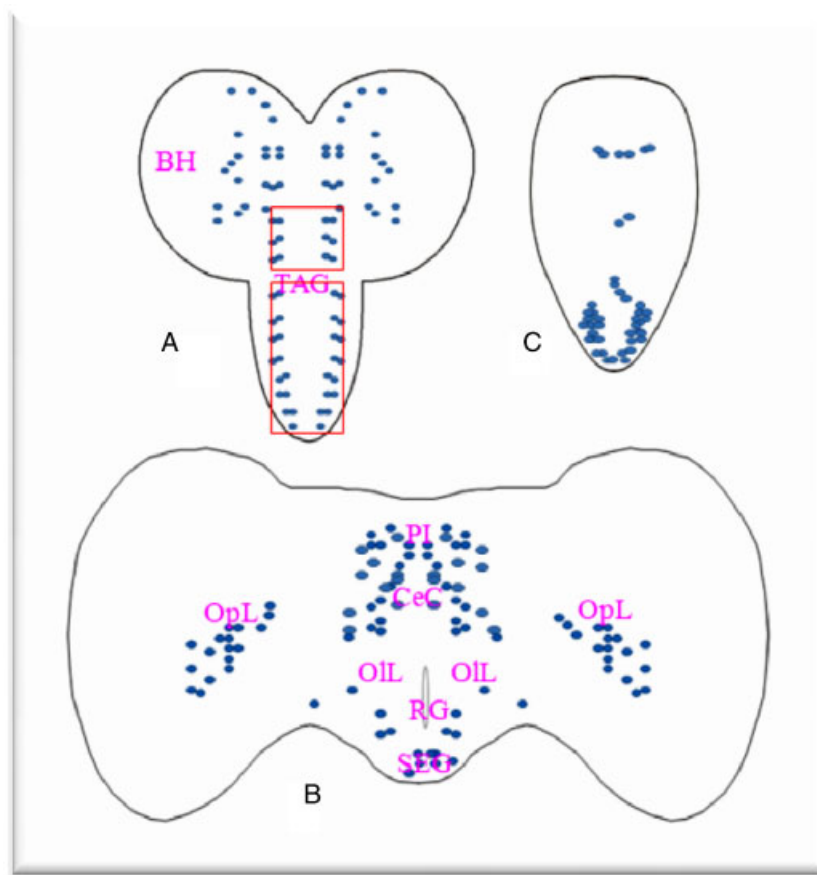


Figure 10. Schematic sketch of the CNS in larval and adult stages of the stable fly showing the cell distribution patterns of 5-HT IR neurons. Axons with their arborizations from these cells are not shown. (A) A larval CNS including brain hemispheres and TAG. (B) An adult brain. (C) An adult TAG. BH, brain hemispheres. CeC, central complex. OIL, olfactory lobe. OpL, optic lobe. PI, pars intercerebralis. RG, ring gland. SEG, subesophageal ganglion. TAG, thoraco-abdominal ganglion.

CeC contained 50–90 5-HT IR neurons. The SG had approximately 16–22 5-HT IR neurons. Up to 20 5-HT IR neurons were observed in each OpL, but 5-HT IR neurons were not observed in the OIL. The pattern of 5-HT IR neurons in the adult stable fly CNS is similar to the one observed in the blood-feeding bug, *Rhodnius prolixus* (Lange et al., 1988).

In the larval TAG, there were three pairs of 5-HT IR neurons on each side. They appeared as symmetrical pairs in the TAG mid-ventral area (Fig. 10A square), which coincided with the prothorax, mesothorax, and metathorax. In the adult TAG, three clusters of 5-HT IR neurons can be seen in the thoracic neuromere region (Figs. 7B and 10C). Three thoracic neuromeres were observed in adult flies (Fig. 7B). Seven pairs of 5-HT IR neurons and one unpaired were distributed symmetrically on each side of the anterior-lateral margin of the larval TAG (Fig. 10A rectangle). 5-HT IR neurons in the adult TAG are too close to count individually. However, the 5-HT IR neurons are visible in three thoracic neuromeres and eight abdominal neuromeres (Figs. 7B, D and 10C). Both the number and pattern of 5-HT IR neurons in the CNS

of the larva are similar to those observed in *D. melanogaster* (Valles and White, 1987; Lee and Hall, 2001).

5-HT IR Patterns in the CPS

Neuroactive molecules play key roles in the blood-feeding process. The cibarial pump of Muscoid flies helps draw blood through the food canal (Elzinga and Broce, 1986). Reid (1994) reported cibarial armature in adult female blackflies, suggesting cibarial pump of nematoceran adult female flies helps draw blood through the food canal. Davis (1985) described 5-HT innervations associated with the mouthparts of *Periplaneta Americana*. Lange et al. (1989) reported on such innervations in the feeding processes of *R. prolixus*. The cibarial pump is a critical component of the feeding system for blood ingestion in biting flies, including the stable fly. The cibarial pump forms the vacuum which draws blood through the food canal between the labrum and labium (Elzinga and Broce, 1986). Innervations deliver impulses enabling the mouth hooks and CDM to move during feeding. Contractions of the CDM draw food into the mouth cavity (Schoofs and Spieß, 2007).

Structural changes observed in fine structures related to stable fly feeding were associated with metamorphosis from the larval to adult stages, which reflect functional adaptation to different feeding habits throughout the stable fly's life cycle. The difference in both the intensity and the terminal structure of the neuronal processes that innervated cibarial muscles in the immature and adult stages of the stable fly suggested significant changes or reorganization in the feeding-related neuromuscular system. These observations constitute the first detailed account of the fine structures of the cibarial pump and innervation of 5-HT IR neurons in both the immature and mature stages of a biting fly species. Nerves arising from the brain were found to be associated with appendages and muscles of the feeding system. Evidence of a serotonergic system is present in the mouthparts, particularly the cibarial pump. Based on this finding, we can hypothesize that 5-HT plays a crucial role in stable fly feeding behavior. Further behavioral and pharmacological experiments are necessary to test such hypothesis. The involvement of 5-HT in insect feeding behavior has been documented in the flesh fly, *Neobellieria bullata* (Parker) (Dacks et al., 2003).

5-HT IR Patterns in the Partial Digestion Systems

5-HT has been studied in the digestive system of several organisms including the blowfly *Calliphora vomitoria* (Berridge, 1972; Trimmer, 1985) and the yellow fever mosquito *A. aegypti* (Novak et al., 1995). Our observations indicate that nerve junctions originate from the brain and run down to the foregut and the midgut, which comprise the stomatogastric nervous system. Intense 5-HT immunoreactivity was observed in processes surrounding the proventriculus in larvae, and processes with 5-HT immunoreactivity projected from this area and extended from the proventriculus to the surface of the anterior midgut. The bulb-like proventriculus in larvae metamorphosed into a PR in adult stable flies. A strong signal of 5-HT immunoreactivity was detected in the neural network associated with the ring proventriculus of adult stable flies. Serotonin (5-HT) IR nerves extended along the DD and midgut.

In summary, we have identified and localized 5-HT immunoreactivity in the feeding system of the stable fly through immunohistological techniques. Additionally, neuroanatomical and physiological aspects involving 5-HT in stable fly larvae and adults were described. These findings suggest that 5-HT plays a role in regulation of

the feeding process in stable flies. Blood feeding is a key behavior of adult stable flies and an attractive target for disruption as an avenue for effective control of this important pest affecting animals and humans. Understanding the physiology and molecular biology of the stable fly is necessary for developing novel fly control technologies (Bram, 1992). New molecular targets, including 5-HT receptors, which are critical for neural signaling are being sought for developing the next generation of pesticides (Wolstenholme et al., 2007; Cai et al., 2010). For example, insecticides with novel mode of action, such as flonicamid and pymetrozine, have been developed commercially as feeding blockers for the control of plant sucking insects (Kaufmann et al., 2004; Morita et al., 2007). Further study of the functions of 5-HT and its receptors in relation to blood feeding in the stable fly would help uncover new molecular targets that can be used to develop novel insecticides or anti-feeding agents for the control of this economically important insect.

ACKNOWLEDGMENTS

The authors thank Drs. Kevin Temeyer and Pia Olafson and two anonymous reviewers for critical review of the manuscript, Darci Burchers for excellent technical support in confocal Imaging, and Matthew Waldon for maintaining the stable fly colony. S.S.L. is supported by a USDA, ARS, Postdoctoral Research Associate Program award to A.Y.L.

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